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Heme oxygenase-1 induction through p38 MAPK/Nrf2 activation by ethanol extract of *Artemisia capillaries* inhibits LPS-activated iNOS, COX-2, and HMGB1 in RAW 264.7 cells

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Abstract

Heme oxygenase-1 (HO-1) negatively regulates inflammatory cytokines in lipopolysaccharide (LPS)-activated RAW 264.7 cells. The purpose of the study was to know whether anti-inflammatory effect of the extract of *Artemisia capillaries* (EAC) is responsible for HO-1 protein expression in RAW264.7 cells. EAC increased HO-1 expression in a time and concentration-dependent manner and inhibited the expression of iNOS (NO) and COX-2 (PGE_2), and release of high mobility group box 1 (HMGB1) in LPS-activated RAW 264.7 cells, respectively. EAC also inhibited NF-kB activity in LPS-activated cells. Finally, the induction of HO-1 as well as inhibition of iNOS and COX-2 by EAC was inhibited by SB203580 but not by SP600125, PD98059, nor LY294002. These results suggest that p38 MAPK/Nrf2-dependent HO-1 induction by EAC is at least rationalized for traditional use of this herb to treat in inflammatory disorders.

Keywords: Heme oxygenase, *Artemisia capillaries,* inflammation, p38MAPK, RAW 264.7 cells

Introduction

Heme oxygenase 1 (HO-1) degrades heme molecule into ferrous iron, carbon monoxide (CO) and biliverdin which is ultimately converted to bilirubin (BR) by biliverdin reductase (Otterbein et al., 1999; Ryter et al., 2006). It has been shown that HO-1 and its by-products play a role in a wide range of actions that could be important during the solution phase of inflammation, with macrophages acting as the pivotal target (Otterbein et al., 1999; Ryter et al., 2006). Administration of HO-1 inducible agents inhibits the expression of the inflammatory genes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in macrophages and subsequently decreases NO and PGE_2 production (Tsoyi et al., 2008). High mobility group box 1 (HMGB1), a nonhistone DNA-binding molecule, has been

reported to be accumulated in the systemic circulation during sepsis in animals or patients in disseminated intravascular coagulation, leading to multiple organ collapse and a successive lethal outcome (Wang et al., 1999; Yang et al., 2004; Tsoyi et al., 2009). Endotoxin (LPS), a cell wall component of Gram-negative bacteria, is a major mediator of sepsis-induced liver damage, multi-organ failure, and chronic liver disease. Owing to the portal blood supply arriving from the intestines and its unique microcirculation, the liver is exposed to high concentrations of nutrients and gut-derived substances including LPS. Recently, we and others reported that HO-1/CO significantly reduces HMGB1 release in lipopolysaccharide (LPS)-activated macrophages in vitro and in the circulation as well as in tissues of septic animals in vivo (Tsoyi et al., 2009; Takamiya et al., 2009). Although *Artemisia capillaries* Thunberg (Compositae) has been widely used as a traditional medicine for liver diseases, such as hepatitis, jaundice, and fatty liver (Han et al., 2005), no report has been available for this protective action is associated with HO-1 induction. Thus, it is of great interest to investigate whether HMGB1 and other inflammatory mediators (NO, PGE_2) are affected by EAC, because it could give clue to rationalize why EAC and its active components are used for hepatic disorders for such a long time in traditional medicine. Therefore, present study tested whether ethanol extract of *Artemisia capillaries* (EAC) induces HO-1 induction, which is responsible for, at least, the inhibitory expression of pro-inflammatory genes in LPS-activated macrophages. Since HO-1 couples the activation of mitochondrial biogenesis to antiinflammatory cytokine expression, it significantly reduces inflammatory cytokines including HMGB1, a critical mediator of death in sepsis (Tsoyi et al., 2011) and liver diseases (Ogiku et al., 2011; Zhou et al., 2012; Oshima et al., 2012). We reports here that ECA induces HO-1 protein expression in RAW 264.7 cells via the p38MAPK/Nrf-2 pathways. Importantly, EAC inhibited HMGB1 release, and expression of iNOS and COX-2 in LPS-activated macrophages *in RAW 264.7 cells activated with LPS.* Thus it can be explained at least in part the rationale why this herb has been used for treatment of inflammatory diseases such as liver disease.

Materials and methods

Pant material

We previously reported the extraction procedure of this plant (Kwon et al., 2011). In brief, the whole plants of *A. capillaris* was purchased in the local retailer and authenticated by Prof. J. H. Lee (Dongguk University, Korea). The whole plants were dried and grinded to powder. Then the dried powder was extracted with 70% aqueous ethanol to give the 70% ethanol extract (EAC). The voucher specimen (No. 20090920) was deposited in our laboratory. Dulbecco's modified Eagle's medium (DMEM), fatal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. Primary antibodies for HO-1, COX-2, Nrf2, PCNA and responsible secondary antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for β-actin were from Sigma-Aldrich (St. Louis, MO) and iNOS were purchased from BD bioscience (San jose, CA). Small interfering RNA for siHO-1, siNrf2 were purchased from Santa Cruz biotechnology (Santa Cruz, CA) and scramble siRNA was purchased from Invitrogen (Carlsbad, CA). Enzyme-linked immunosorbent assay (ELISA) kit for PGE_2 was purchased from R&D system (Minneapolis, MN).

Cell culture

RAW264.7 macrophages were maintained at 5 x 10^5 cells/ml in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (4.5 mg/ml), glucose (4.5 mg/ml) and incubated at 37℃ in a humidified atmosphere containing 5% CO_2 and 95% air.

Western blot analysis

Western blot analysis was performed by lysing cells in RIPA buffer (25 mM Tris-HCl; pH 7.4, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor mixture. Protein concentration was determined using Bradford assay; absorbance of the mixture at 595 nm was determined with an ELISA plate reader. An equal amount of protein for each sample was resolved using 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a PVDF western blotting membranes (Roche, Germany). The membrane was blocked with 5% skim milk and sequentially incubated with primary antibody and horseradish peroxidase-conjugated secondary antibody followed by ECL detection (Animal Genetics).

Transfection

RAW264.7 macrophages were seeded into 60 mm cell culture dishes at 3 x 10^5 cells /dish 18-24 h prior to transfection. Cells were transfected with 2 ug NF-kB luc vector or 100 nM siHO-1 RNA, 100 nM scramble siRNA using the SuperFect fragment (Qiagen, Valencia, CA). After incubation for 4 h, the medium was replaced with fresh medium.

NOx measurement

The nitrite and nitrate (NOx) concentration in the medium was measured, as an indicator of NO production, according to Kang et al (1999). One hundred μl of each supernatant was mixed with the same volume of Griess reagent; absorbance of the mixture at 545 nm was determined with an ELISA plate reader.

PGE_2 measurement

RAW264.7 macrophages were cultured in 60 mm cell culture dish, pre-incubated for 1 h with different concentrations of EAC and then stimulated for 16 h with LPS. One hundred μl of supernatant of culture medium was collected for the determination of PGE_2 concentrations using the ELISA kit.

Cell cytotoxicity

For determination of cell viability, 50 mg/ml of Thiazolyl Blue Tetra-zolium bromide (MTT) was added to 1ml of cell suspension (3 x 10^5 cells/ml in 24-well plates) for 4 h, and the formazan formed was dissolved in DMSO; optical density was measured at 570 nm.

siRNA and vector transfection

The RAW264.7 macrophages were seeded into 60mm cell culture dishes at 5 x 10^5 cells/dish 18–24 h prior to transfection. The cells were transfected with 2 mg NF-κB luciferase vector or 100 nM siHO-1 RNA, 100 nM scramble siRNA using the SuperFect® fragment (Qiagen, Valencia, CA). After 4 h incubation, the medium was replaced with a fresh one.

NF-kB luciferase activity

After experimental treatments, cells were washed twice with cold PBS, lysed in a lysis buffer provided in the dual luciferase kit (Promega, Madison, WI), and assayed for Luciferase activity using a TD-20/20 luminometer (Tumer Designs, Sunnyvale, CA), according to the manufacturer's protocol. All transfections were done in triplicate. The data are presented as a ratio between Firefly and renilla luciferase activities.

Statistical evaluation

Scanning densitometry was performed using an Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA). Data are expressed as the mean ± SD of results obtained from the number of replicate treatments. Differences between data sets were assessed by one-way analysis of variance followed by Newman-Keuls tests. $P < 0.05$ was accepted as statistically significant.

Results

Effect of EAC on cell viability

Trypan blue assay was performed after 24 h incubation to test cell viability after cells were treated with different concentrations of EAC. When performed cell viability by MTT assay almost identical results were obtained (data not shown). As shown in figure 1, no significant toxic sign was observed until 100 μg/ml of EAC. However about 20% of survival rate was observed at dose of 500 μg/ml EAC. Therefore, through the entire experiments, maximum concentration of EAC was no higher than 100 μg/ml.

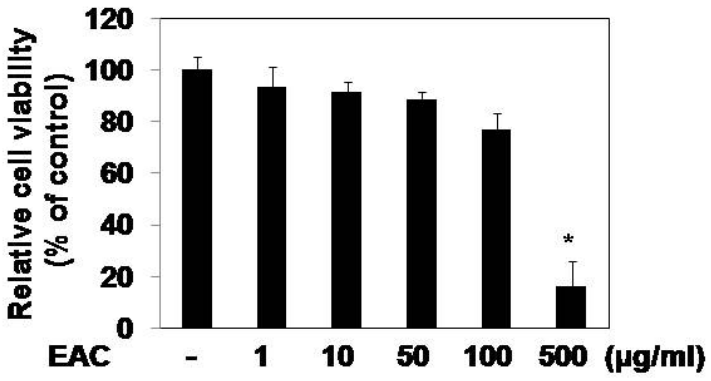

Figure 1. Cytotoxic effect of EAC in RAW264.7 cells. Cell viability was determined by MTT. Cells were treated with different concentrations of ECA for 24 h. The results are expressed as means ± SE of three independent experiments. *$p < 0.05$ significantly different from control.

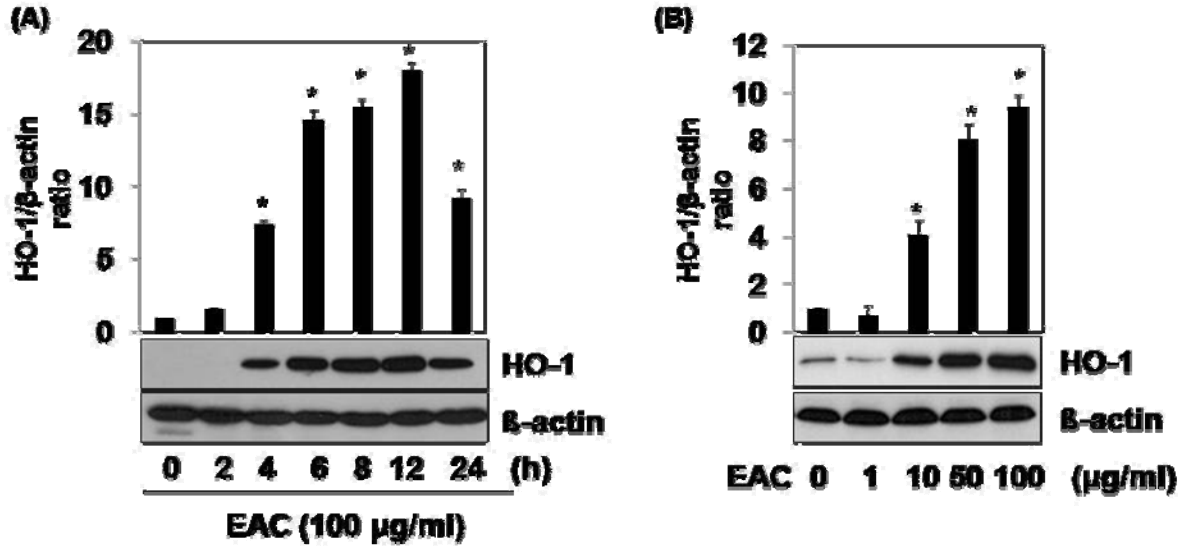

Figure 2. Effect of EAC on HO-1 expression. Expression of HO-1 by EAC was examined in a time-dependent manner with 100 μg/ml ECA (A) and concentration-dependent manner with different doses after 8 h treatment (B). After completion, proteins were extracted and western blot analysis was performed using anti-HO-1 antibody. The results are expressed as means ± SE of three independent experiments. * $p < 0.05$ compared with untreated cells.

EAC increases HO-1 expression

Figure 2 shows that EAC increases HO-1 protein expression in a time- and concentration-dependent manner in RAW 264.7 cells. When used a fixed concentration of EAC (100 μg/ml), HO-1 expression began to appear as early as 4 h and continuously increased until 12 h and then diminished at 24 h (a). Fig.2b shows that EAC increases HO-1 protein in a concentration-dependent mode.

EAC induces HO-1 expression by Nrf2 translocation

Nrf2 plays an important role in the induction of HO-1 in many cells including macrophages. We asked whether EAC-mediated HO-1 induction is related to activation of Nrf2. As shown Figure 3a, Nrf2 retained in cytosol fraction in untreated cells, which was translocated to nuclear fraction by increasing concentration of EAC. Almost all Nrf2 were translocated to nuclear from cytosol fraction at concentration exceeding 50 μg/ml of EAC. To further confirm EAC activates Nrf2 translocation, we used small interference RNA technique. As expected, the increased HO-1 induction by EAC was diminished in siNrf2-transfected cells (Fig.3b).

EAC inhibits iNOS/NO and COX-2/PGE_2 in LPS-activated macrophages

Figure 4a shows that EAC concentration-dependently inhibited expression of iNOS protein and production of NOx in LPS-stimulated macrophages. LPS, bacterial endotoxin, significantly upregulated iNOS protein and subsequently increased NO production. However, in the presence of EAC, upregulation of iNOS by LPS was significantly inhibited. For example, 10 μg/ml EAC decreased NO production about 50%. COX-2 enzyme is also important

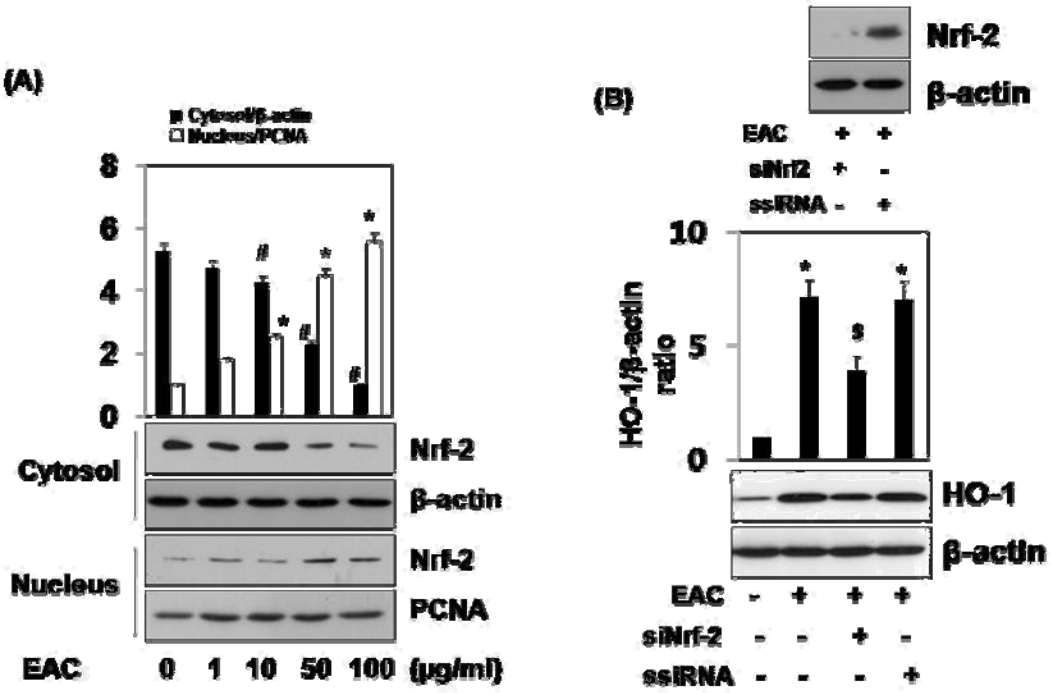

Figure 3. Effect of EAC on Nrf2 translocation in macrophages. EAC was treated with different concentrations (1, 10, 50, 100 μg/ml) for 1 h. Nuclear extracts were subjected to SDS-PAGE, and Nrf2 was detected by immunoblotting (A). To see Nrf2 is involved in HO-1 induction by EAC, cells were transfected with either siNrf2 RNA or scramble siRNA (ssiRNA), where transfection efficacy was confirmed by expression of Nrf2 with EAC (B). The siNrf2 RNA- or ssiRNA-transfected cells were treated with EAC and incubated for 8 h, which was subjected to western blot analysis using anti-HO-1 antibody. The results are expressed as means ± SE of three independent experiments. $^{*,\#}$ $p < 0.05$ compared with untreated cells, respectively. $^{\$}$ $P < 0.05$ compared with ECA-treatment.

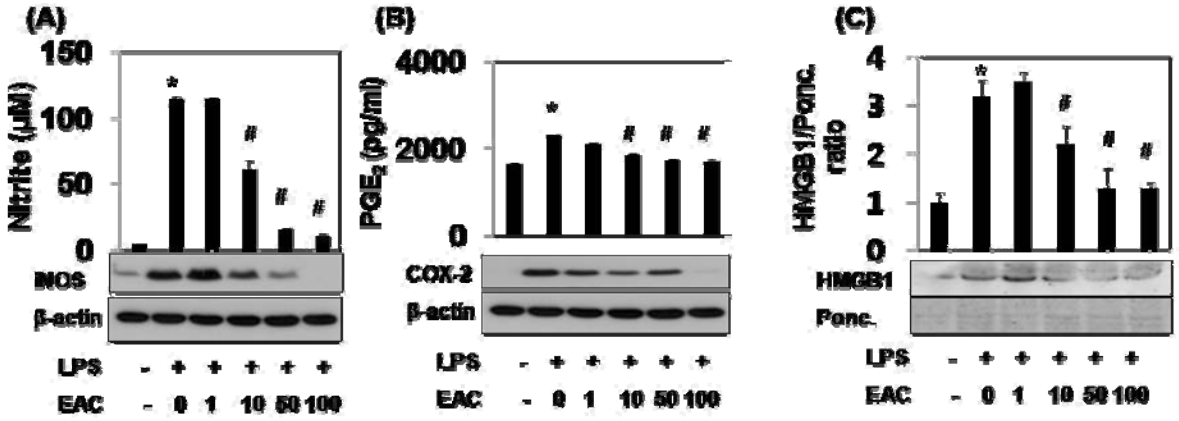

Figure 4. Anti-inflammatory effect of EAC in LPS-activated macrophages. Cells were stimulated with different concentrations of ECA for 1 h prior to LPS (1 μg/ml) and incubated for 8 h for detection of iNOS and COX-2 or 16 h for HMGB1. Proteins were extracted and subjected to western blot analysis for iNOS (A), COX-2 (B) protein, and HMGB1 (C). The production of NO (A) and PGE_2 (B) from the culture medium was also measured as described in Methods. After 16 h incubation, culture media were concentrated and analyzed by western blot for detection of HMGB1(C). The results are expressed as means ± SE of three independent experiments. * $p < 0.05$ compared with untreated cells. $^{\#}$ $p < 0.05$ compared with LPS-treated cells.

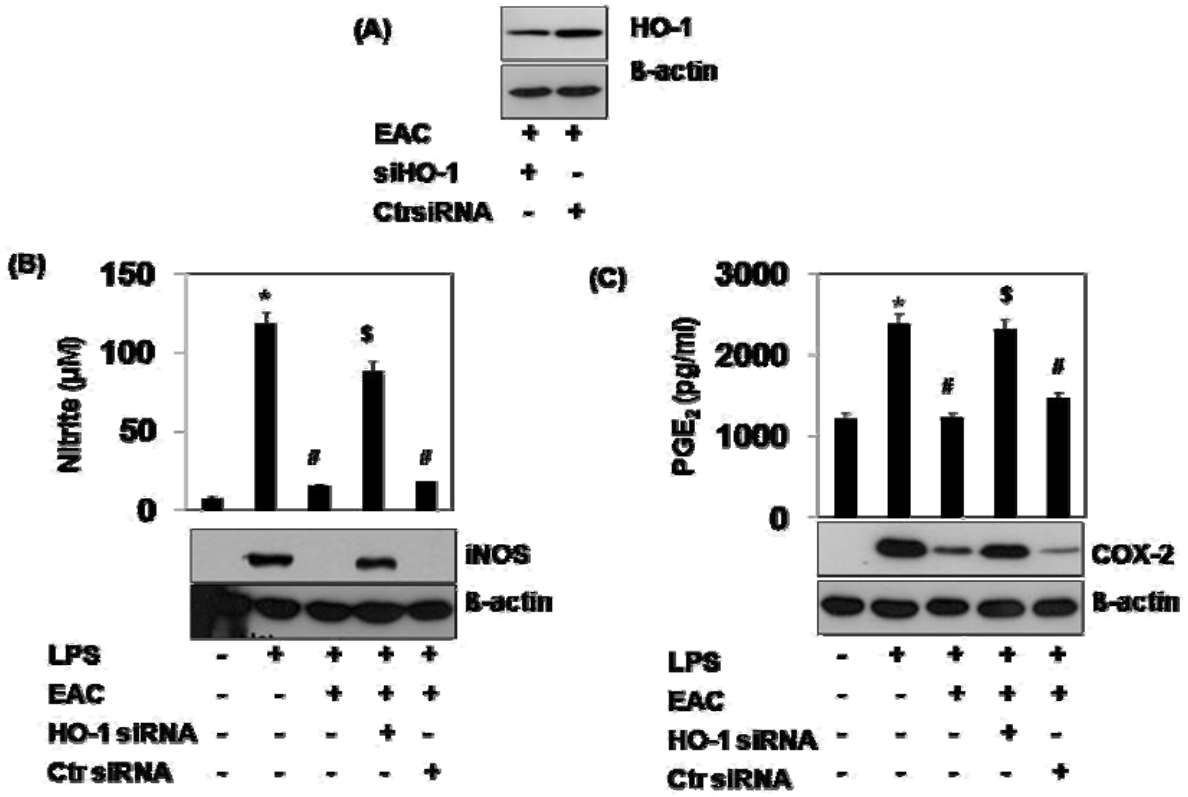

Figure 5. Effect of EAC on expression of iNOS and COX-2 in HO-1 siRNA transfected macrophages. Cells were transfected with either siHO-1 RNA or scramble siRNA (ssiRNA), where transfection efficacy was confirmed by expression of HO-1 with EAC (A). The siHO-1RNA- or ssiRNA-transfected cells were treated with ECA and incubated for 8 h, which was subjected to western blot analysis using anti-iNOS (B) or anti-COX-2 antibody (C), respectively. The results are expressed as means ± SE of three independent experiments. * $p <$ 0.05 compared with untreated cells. $^{\#}$ $P < 0.05$ compared with LPS-treatment. $^{\$}$ $P < 0.05$ compared with EAC+ LPS-treatment.

mediator of inflammation. As expected, EAC also significantly and concentration-dependently decreased the expression of COX-2 and production of PGE_2 (Fig.4b). Interestingly, the concentration of EAC that reduces iNOS and COX-2 also significantly reduced LPS-induced HMGB1 release (Fig.4c). This effective concentration is well correlated with the concentration that significantly induces HO-1 by EAC.

HO-1 is crucial for anti-inflammatory effect of EAC

As to whether HO-1 induction by EAC is really critical for anti-inflammatory action, we investigated expression of HO-1, iNOS, and COX-2 protein using siHO-1 RNA transfected cells. After confirming the transfection efficacy of siHO-1 RNA as shown in Fgi.5a that EAC-mediated HO-1 induction was inhibited by siHO-1-transfected cells but not control siRNA-transfected cells. We also found that the diminished expression of iNOS/NO (Fig.5b) and COX-2/PGE_2 (Fig.5c) by EAC was reversed by siHO-1 RNA transfected cells, indicating that HO-1 enzyme plays a key role for anti-inflammatory action of EAC.

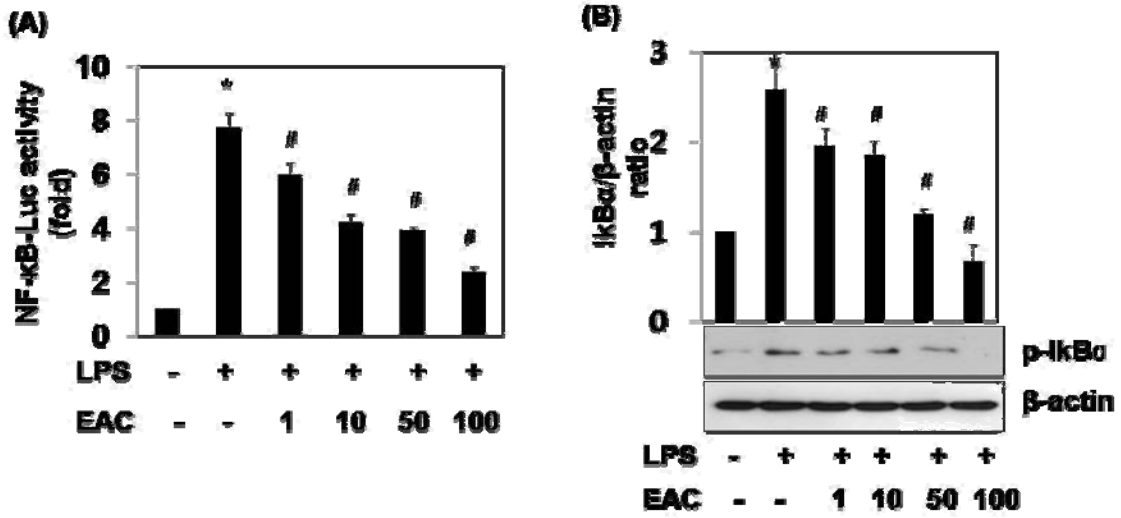

Figure 6. Inhibition of NF-κB activation by EAC in RAW 264.7 cells. NF-kB luciferase activity was measured in cells transiently transfected with NF-kB luciferase(A). Cells were pretreated with EAC (1, 10, 50, or 100 μg/ml) for 1 h and then incubated for another 1 h with LPS (1 μg/ml). After treatment, phosphor-IκBα (B) levels were determined by western blot analysis. The results are expressed as means ± SE of three independent experiments. * $p < 0.05$ compared with untreated cells. # $p < 0.05$compared with LPS-treated cells.

EAC inhibits NF-kB activity

NF-kB is well known transcription factor for inflammation. To understand whether EAC inhibits NF-kB activity, we measured lucifease activity assay as described in Method. Figure 6a clearly shows that EAC concentration-dependently inhibited LPS-induced NF-kB lucifearase activity. To further confirm this result, when measured phosphorylation of IkBa expression, EAC also concentration-dependently diminished the p-IkB level (Fig.6b).

The signal mechanism responsible for induction of HO-1 by EAC

Finally, we asked the mechanism by which EAC induces HO-1 in macrophage cells. p38/Nrf-2 pathway has been reported to induce HO-1 (Tsoyi et al., 2010). Fig. 7a shows that SB203580, p38 MAPK inhibitor, strongly reduced HO-1 induction by EAC. However, SP600125, PD98059, or LY294002 did not reduce HO-1 expression by EAC. To confirm that p38 MAPK is involved in HO-1 induction by EAC, we investigated the expression of iNOS and COX-2 in the presence of different MAPK inhibitors. Fig. 7b shows that increased iNOS and COX-2 by LPS was reduced by EAC, where only SB203580 reversed the effect of EAC.

Discussion

In the present study, we explored whether anti-inflammatory action of EAC is at least related with upregulation of HO-1 in RAW 264.7 cells. We clearly demonstrated that EAC increased HO-1 expression in RAW 264.7 cells in a concentration- and time-dependent manner. Indeed, HO-1 is the inducible isoform of HO (Maines, 1988) which now recognized as an important anti-inflammatory enzyme featured by its antioxidant activity (Ewing et al.,

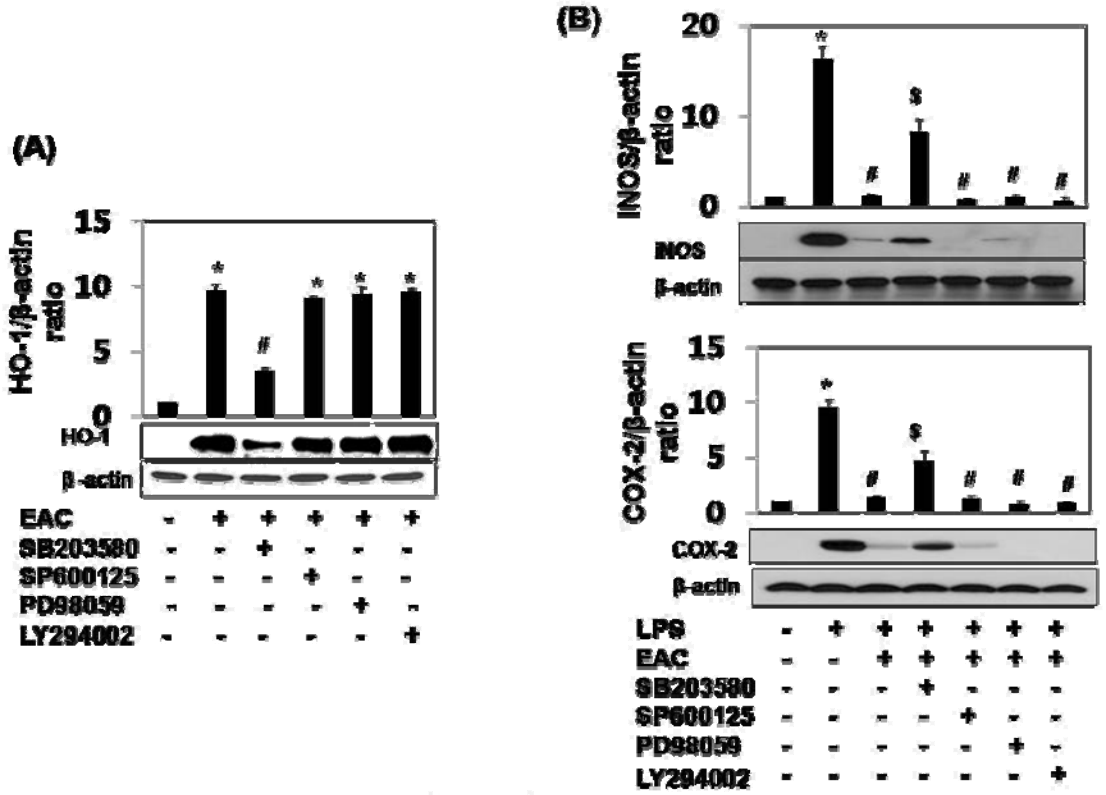

Figure 7. Signaling pathways of EAC on HO-1 expression. Cells were exposed to SB203580 (10 μM), SP60-0125 (40 μM), PD98059 (50 μM), or LY294002 (10 μM) for 1 h, and stimulated with EAC (100 μg/ml) for 8 h. Then, HO-1 protein level was determined by western blot analysis (A). Cells were pretreated with various signal inhibitors for 1 h prior to EAC treatment. Then cells were exposed to LPS for 8 h (iNOS, COX-2) and western blot was performed using corresponding antibodies, respectively (B). Production of nitrite and PGE_2 was measured as described in Methods. The results are expressed as means ± SE of three independent experiments. $^{*}p < 0.05$ compared with untreated cells. $^{\#}p < 0.05$ compared EAC- or LPS-treated cells. $^{\$}p < 0.05$ compared EAC+ LPS-treated cells.

1993; Presteraet al, 1995; Kutty t al., 1995). That the induction of HO-1 can prevent or mitigate the symptoms associated with related ailments has increasingly demonstrated in experimental models of acute inflammation (Kobayashi et al., 2006). Then, what is the possible mechanism for induction of HO-1 by EAC? In general, induction of HO-1 occurs to defend the cell from the harsh environment. Because activation of MAPKs plays a central role for the induction of HO-1 gene expression (Alam and Cook, 2007), and JNK and p38 are primarily induced by stress-related stimuli (Kyriakis and Avruch, 2001; Wagner and Nebreda, 2009), it is of great interest to investigate which MAPKs are responsible for EAC-mediated HO-1 induction. We found that SB203580 (p38 MAPK inhibitor), but neither SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), nor LY294002 (PI3K inhibitor) significantly inhibited EAC-induced HO-1 induction, indicating that p38 MAPK plays a crucial role for HO-1 induction. Indeed, numerous reports suggested that activation of p38 MAPK mediates an anti-inflammatory action. Thus, involvement of p38 MAPK is well documented in many diseases, including inflammatory disorders. Especially, it has been shown that CO, one of the byproducts of HO-1, provides an anti-inflammatory effect through activation of p38 MAPK (Brugger et al., 2010; Kohmoto et al., 2007; Schwer et al., 2010). Because both induction of HO-1 and inhibition of pro-inflammatory gene expression of EAC

were reversed by the presence of SB203580, we believe that p38 plays a key role for EAC-mediated HO-1 induction which is in agreement with others (Wijayanti et al., 2004). Recently, we reported that ethyl pyruvate, a potent anti-inflammatory agent, induced HO-1 through a p38 MAPK- and NRF2-dependent pathway by decreasing GSH cellular levels (Jang et al., 2012). Thus, it needs further study whether EAC also depletes cellular GSH levels activating p38MAPK to induce HO-1.

Although the precise mechanism of HO-1 induction by EAC remains to be explored, we clearly demonstrated that anti-inflammatiory action of EAC mediated through HO-1. For instance, we demonstrated that Nrf2-mediated HO-1 upregulation by EAC reduced iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells, which was reversed in siHO-RNA transfected cells, indicating that HO-1 is crucial for anti-inflammatory action by EAC. NO is recognized as a mediator and regulator of inflammatory response and is produced in high amounts by iNOS in activated inflammatory cells (Luss et al., 1994; Galler et al., 1994). COX-2 also can be affected directly at its enzymatic activity by nitric oxide and iNOS (Vane et al., 1994). However, it should be noted that p38 MAPK activation found to be critically involved in LPS-induced iNOS expression and NO release in RAW 264.7 macrophages (Chen et al., 1999). Although we cannot properly explain this discrepancy at the present time, the concentration of the p38 MAPK inhibitor (SB203580) or different methods or animal models may have caused this difference.

Several studies suggested that the signaling pathways for HO-1 induction are also dependent on NF-kB signal (Wijayanti et al., 2004). NF-κB stimulates the expression of enzymes whose products contribute to the pathogenesis of the inflammatory process, including iNOS and COX-2 (Pahl, 1999; Luss et al., 1994; Galler et al., 1994; Vane et al., 1994). Moreover, induction of NF-κB during liver injury has also been reported in hepatocytes of rat (Orfila et al., 2005). Endotoxin (LPS), a cell wall component of Gram-negative bacteria, is a major mediator of sepsis-induced liver damage, multi-organ failure, and chronic liver disease. Owing to the portal blood supply arriving from the intestines and its unique microcirculation, the liver is exposed to high concentrations of nutrients and gut-derived substances including LPS. LPS is a strong stimulator to activate NF-kB in many cells including RAW 264.7 cells (Tsoyi et al., 2009). We found that EAC dose-dependently inhibited NF-kB activity by showing of concentration-dependent inhibition of phosphorylation of IkBa and luciferase activity in cells activated with LPS. Lastly, we recently reported that HO-1 inhibits NF-kB activity which is associated with inhibition of translocation of HMGB1 in LPS-activated macrophages (Ha et al., 2011). In addition, increased plasma HMGB1 levels were significantly reduced by HO-1 inducer (Tsoyi et al., 2011). Inhibition of production of HMGB1 by Kupffer cells by glycyrrhizin, a natural anti-inflammatory triterpene, has been demonstrated to protect liver injury caused by ischemia and reperfusion (Ogiku et al 2011). In this regard, we showed that EAC inhibited LPS-activated HMGB1 in RAW 264.7 cells. Thus, it is highly speculated that EAC may be beneficial in liver disease, although it warrants by in vivo experiment.

In summary and conclusion, we investigated anti-inflammatory action of EAC in association with induction of HO-1. The present results show that EAC increased HO-1 induction via p38MAPK/Nrf2 signals, which is critical for anti-inflammatory action as evidenced by inhibition of LPS-activated iNOS, COX-2, and HMGB1 release in RAW 264.7 cells.

Thus, induction of HO-1 may be an important mechanism of action for anti-inflammatory action of EAC.

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